

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 6 : C12N 1/20, A61K 39/02, 39/112, 39/108, 39/106, 39/07 // (C12N 1/20, C12R 1:01) (C12N 1/20, C12R 1:19) (C12N 1/20, C12R 1:42) (C12N 1/20, C12R 1:085)</p>	<p>A1</p>	<p>(11) International Publication Number: <b>WO 98/02523</b>  (43) International Publication Date: 22 January 1998 (22.01.98)</p>
<p>(21) International Application Number: PCT/GB97/01932 (22) International Filing Date: 15 July 1997 (15.07.97)  (30) Priority Data: 9615022.2 17 July 1996 (17.07.96) GB  (71) Applicant (for all designated States except US): THE MIN- ISTER OF AGRICULTURE FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall Place, Lon- don SW1A 2HH (GB).  (72) Inventors; and (75) Inventors/Applicants (for US only): BARROW, Paul, Andrew [GB/GB]; The Former Vicarage, Letcombe Hill, East Challow, Wantage, Oxon OX12 9RP (GB). LOVELL, Margaret, Ann [GB/GB]; 82 Dibleys, Blewbury, Oxon OX11 9PU (GB). TURNER, Arthur, Keith [GB/GB]; 2 Carse Close, Abingdon, Oxon OX14 2RA (GB).</p>	<p>(74) Agent: SKELTON, S., R.; D/IPR, Formalities Section (Pro- curement Executive), Poplar 2, MOD Abbey Wood #19, P.O. Box 702, Bristol BS12 7DU (GB).  (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
<p>(54) Title: VACCINE PREPARATIONS  (57) Abstract  The invention provides a live gut-colonising microorganism (e.g. <i>Salmonella</i>) capable of evoking an immune response in an animal (e.g. a chicken) for use as a vaccine, characterised in that the microorganism has a specific mutation which impairs its ability to colonise the alimentary tract of the animal. Preferably the microorganism is attenuated, and/or has a negative serological marker, and/or expresses antigens from heterologous organisms. Preferably the mutation comprises the inactivation of a gene selected from: <i>hupA</i>, <i>dxsA</i>, <i>rfaY</i>, <i>sipC</i> or <i>clpB</i>. Also disclosed are associated methods generating the vaccines, pharmaceutical preparations, methods of protecting animals and the products of these methods.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakistan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

VACCINE PREPARATIONS

The present invention relates to live vaccines, methods of generating them and applications for them. The invention further relates to genes and polynucleotides which may be used to  
5 manipulate such vaccines.

Poultry is a significant source of food-poisoning organisms, including *Salmonellas*, in man. Poisoning occurs following ingestion of *Salmonella* organisms with poultry meat. Contamination of the meat often stems from contamination of the  
10 carcass with faecal material.

Additional contamination can occur when highly invasive strains penetrate the alimentary mucosa and become localised in internal organs used in cooking, such as the liver, heart or ovaries (which can also produce infected eggs). Most serotypes, however,  
15 are confined to the alimentary tract of poultry and only exceptionally produce clinical disease in the bird.

Present methods for controlling faecal shedding of *Salmonella* serotypes by commercial poultry, or for preventing initial infection, are either inadequately carried out, not completely  
20 effective or produce logistical problems. With the technology and information already available it would be feasible to rear chickens completely free of *Salmonella* and also *Campylobacter*. However, the hygiene and management restrictions would place considerable constraints on an industry with already small profit  
25 margins.

Another approach has been antibiotic therapy to rid the poultry of infection. However this can induce antibiotic resistance in the *Salmonella* organisms themselves or in others such as *Escherichia coli* and *Campylobacter*. Antibiotics may also be  
30 relatively ineffective in reducing faecal excretion.

Competitive exclusion of undesirable microorganisms can be used on its own in newly hatched broilers or in conjunction with antibiotics in breeders. This has been applied extensively in some countries, but is not yet used in many parts of the broiler

industry. There are two standard methods whereby this can be done. The first is based upon the fact that newly hatched chicks are far more susceptible to *Salmonella* infection than are adult birds, the difference being the result of the normal adult  
5 intestinal microbial flora possessed by the adult. Administration of this flora to chicks gives them, within a few hours, the full resistance of the adult bird.

The second method utilises bacteria which are *salmonella*-like in their colonisation characteristics but which are avirulent  
10 (Barrow et al., Epidemiol. Infect. 1987, 98, 311-322; and A Berchieri Jnr et al. Epidemiol. Infect. 1990, 104, 427-441).

Immunisation with killed vaccines is used on an *ad hoc* basis with varying degrees of success. However, some objections exist to the use of live, attenuated vaccines with poultry (see Barrow (1990)  
15 "Immunological control of *Salmonella* in poultry" Proceedings of the International Symposium on control of Human Bacterial Enteropathogens in poultry: Atlanta, Georgia, USA, 1989. In particular, because the vaccine organisms are shed in considerable numbers following immunisation, there is a risk that  
20 they will enter the food chain with possible, unknown, effects on consumer. The risk is particularly disturbing when the vaccine contains uncharacterised mutations.

Notwithstanding this a number of live, attenuated vaccines are currently commercially available. These include Zoosaloral H (TM)  
25 from Impfstoffwerk Dessau-Tornau GmbH and also TAD *Salmonella* vac (RTM) T from TAD Pharma.

The general principles of colonisation by microorganisms of the alimentary tract of a host animal have been the subject of a number of papers, particularly as regards *Salmonella* in chickens.

30 It is known that following infection with a *Salmonella* serotype that characteristically produces systemic disease typhoid-like disease, or with a vaccine strain derived from that serotype, a variety of animals develop a strong immunity against reinfection. Such serotypes, which include *S. gallinarum* and *S. cholerae-suis*,

tend to colonise the gut poorly in the absence of clinical disease (e.g. excreted for only 2 to 3 days). A contribution to the relatively poor intestinal colonising ability in chickens by *S. gallinarum* has been shown to be made by the virulence-associated plasmid

In contrast to the above, *Salmonella* serotypes associated with food-poisoning (mainly *S. typhimurium* and *S. enteritidis*) rarely produce systemic diseases unless very young chicks are infected. These serotypes tend to colonise the alimentary tract of poultry well, that is they are excreted in the faeces for many weeks (e.g. 7 to 11 weeks) and in considerable numbers after oral inoculation. Such serotypes are usually isolated from the caeca and, to a lesser extent, the crop.

Some work has been published relating to one mutant of *S. typhimurium* having reduced colonising ability (Craven (1994) Avian Diseases 38:401-408), this work was essentially concerned with the underlying methods by which the bacteria adhere to cecal mucus.

The applicants have found that impaired colonisation is useful in the field of live vaccines.

The present invention provides a live microorganism for use as a vaccine, said microorganism comprising a gut-colonising microorganism which is capable of evoking a protective immune response in an animal to which it is administered, characterised in that the microorganism is a mutant organism whose ability to colonise the alimentary tract of said animal is inhibited as a result of a mutation.

Mutation of a microorganism so as to inhibit the colonisation of the alimentary tract of an animal can be achieved using various techniques. For example, following random mutation, for instance using transposons, suitable mutants can be identified by testing the gut colonising properties as illustrated hereinafter.

In particular, impaired ability of mutants to colonise the alimentary tract of an animal is indicated by the observation

that the mutant microorganisms are excreted in smaller quantities and/or for a shorter time scale following challenge than are the parent strain (i.e. a similar strain which does not include the mutation). This can be assessed without undue burden by the methods employed in "Chromosomal transposon mutations affecting intestinal colonisation of chickens by *S. Typhimurium*" by Barrow and Lovell in CNEVA/INRA Reports and Communications: Salmonella and Salmonellosis (September 15-17, 1992; Ploufragan/Saint-Brieuc-France) or methods analogous to these for non-Salmonella microorganisms. In that study cloacal swabs were taken at various intervals from a statistically significant group following challenge with mutant and parent strains. Swabs were plated out and the presence of salmonella organisms (either as colonies or organisms detected by enrichment culture) was assessed. Preferably the time scale over which excretion (for instance as measured by one of these methods) occurs is reduced by at least 50% or more preferably 60, 70, 80 or 90% compared to the parent strain.

Most preferably the number of chickens excreting the microorganism is reduced to less than 20% of that found with parent strain as measured 3 weeks after inoculation

In general, impairment of the ability of the microorganism to colonise the alimentary tract of an animal will arise as a result of a specific mutation which affects a gene associated with gut colonising activity. In particular, the specific mutation in the microorganism results in the inactivation of one or more genes associated with colonisation of the alimentary tract of the animal.

As used herein, the term 'inactivation' means that the gene function is significantly impaired, for instance by down-regulating, mutating or deleting the gene itself or associated nucleotide sequence elements which control its transcription, translation or translocation within the microorganism. Mutations include point mutations, partial deletion or insertion mutations as would be understood in the art.

Thus the vaccine microorganisms of the present invention differ from the corresponding wild type strain or vaccine or parent strains from which they are derived, in that they have been altered to have a specific mutation which gives them an impaired ability to colonise the alimentary tract of the animal.

By 'alimentary tract' is meant any part of the intestine or the caeca - the main site of colonisation.

Once suitable genes have been identified, mutants of the invention can be produced using recombinant DNA technology as illustrated hereinafter.

Microorganisms used in the present invention are suitably those which cause food poisoning or other pathogenic microorganisms which colonise the gut e.g. enterobacteriaceae such as *Salmonella*, *Yersinia*, *E. coli*, *Campylobacter*, *Listeria*, *Bacillus cereus*, *Shigella* etc. In particular, the bacteria comprise *Salmonella*, *E. coli*, *Campylobacter*, *Listeria* or *Bacillus cereus*. Preferably the microorganism comprises a mutant *salmonella* strain.

Examples of suitable genes which can be mutated so as to inhibit the ability of the microorganisms to colonise the alimentary tract of an animal include *hupA*, *dkSA*, *rfaY*, *sipC* and *clpB*. In particular, the animal will be an animal possessing a normal adult gut flora.

The present inventors have established that inactivation of the following genes in *S. typhimurium* can generate mutants having impaired ability to colonise the alimentary tract of chickens: *hupA*, *dkSA*, *rfaY*, *sipC* and *clpB*. All of these genes have been previously characterised. However their role in colonisation was previously unknown. These genes and suitable mutants are described more fully below.

Gene inactivation may be carried out by a number of techniques such as are well known to those skilled in the art (see the latest Edition of Sambrook, Fritsch & Maniatis "Molecular Cloning: A Laboratory Manual", Cold Spring Harbour Laboratory,



Cold Spring Harbour, N.Y.). One suitable method entails allele exchange between the parent *Salmonella* strain and a suicide vector incorporating a mutated, inactivated, form of the gene.

5 Host animals which could be protected include any animals which are susceptible to colonisation by such microorganisms e.g. mammals such as humans or companion animals. Preferably, though, the animals are food animals such as poultry, cattle, pigs or sheep, in particular poultry such as chickens or turkeys.

10 It has been found that by impairing the ability of the microorganism to colonise the alimentary tract of an animal, the microorganisms are attenuated to some extent. This means that the virulence of the microorganism has been reduced whilst the ability of the microorganism to elicit antibodies against the virulent form has been retained. However, the microorganisms  
15 may include further attenuations, i.e. modifications in other genes which lead to reduced virulence. Suitable further attenuations embrace not only those which are characterised in existing commercially available live vaccines, but also those which may later be characterised. Examples of known attenuations  
20 include mutations in the *aro A*, *gal E* and *pur A* genes. Also known are those which comprise mutations in the electron transport genes.

The use of organisms which colonise the alimentary tract such as salmonella, as 'carriers' for immunogenic proteins which can give  
25 rise to other immunity is well known. In these cases, the carrier microorganism is engineered to express antigens from foreign organisms (e.g. from *Shigella*, cholera, malaria sporozoites). Such vaccines may further include the specific mutation of the present invention. In such cases, it would be  
30 necessary for the attenuation to reduce the virulence of the microorganism without significantly reducing the effectiveness of these antigens.

Preferably the microorganisms are further characterised in that they exhibit a negative serological marker and/or are otherwise  
35 differentiable from parental or wild-type strains. This allows

vaccinated animals to be distinguished by serology from animals which had been infected for example with wild-type strains. Examples of such markers may be roughness, non-flagellation, non-fimbriation.

- 5 One suitable marker antigen which could be disabled is the *Salmonella Enteritidis* Fimbrial Antigen (SEFA) disclosed in WO 92/06197 of M.A.F.F. An alternative marker could be certain *rfa* or *rfb* mutants described hereinafter which allow mutated strains to be recognised serologically as a result of the alteration in
- 10 the lipopolysaccharide coat of the organism. Again however, the mutation should not significantly affect the ability of the microorganism to produce the desired immune response in an animal to which it is administered.

1. Certain mutant gut-colonising microorganisms as used above are novel and these form a further aspect of the invention. In particular the invention provides a mutant gut-colonising microorganism, such as a mutant *Salmonella*, *E. coli*, *Campylobacter*, *Listeria* or *Bacillus cereus*, wherein at least one of the genes selected from *hupA*, *dkxA*, *rfaY*, *sipC* or *clpB* has been down-regulated or inactivated so as to inhibit the ability of the microorganism to colonise the alimentary tract of an animal. These microorganisms may comprise further mutations which attenuate the microorganism and/or provide a negative serological marker as described above.

- Microorganisms of the invention are suitably used in the form of
- 15 pharmaceutical preparations or vaccines. Thus the invention further provides a vaccine comprising a microorganism according to any one of the preceding claims in combination with a pharmaceutically acceptable carrier or diluent. The pharmaceutical preparation or vaccine is preferably in dosage
- 20 unit form, containing an amount of any of the live vaccine microorganisms as described above in a non-toxic quantity which is suitable to evoke a protective response in an animal.

Preferably the dosage unit contains around between  $1 \times 10^7$  and  $1 \times 10^9$ , preferably around  $1 \times 10^8$  colony forming units of the

microorganism in a form adapted for direct administration to the animal, for instance which is suitable for oral administration as a tablet, or in around 0.1-1 ml of an aqueous carrier. A stock suspension containing precise multiples (e.g. 100, 500, 1000) of dosage units forms a further embodiment of this aspect of the invention.

In a further aspect of the invention there is provided a method of protecting an animal comprising administering a live vaccine microorganism as described above to said animal. Depending on the animal it may be advantageous to administer the live vaccine on more than one, for instance two, three or four, separate occasions in order to increase the protective response. In addition, the vaccine may be administered in combination with other killed vaccines which may also increase the response. Preferably the timing of the administration(s) is selected such as to maximise the effect. For instance in vaccinating chickens it may be preferable to give the first administration to chicks as early as possible (e.g. within the first day or few days of hatching). This may encourage a more systemic establishment of the vaccine strain thereby increasing the protective immunogenic response. Additionally early administration may lead to a competitive exclusion effect of non-vaccine organisms.

Preferably the live vaccine microorganism is administered orally to the animal e.g. in drinking water, with feed, or as a spray

A further aspect of the invention provides a method of compromising the ability of gut-colonising microorganisms to colonise the alimentary tract of a animal comprising the specific inactivation of any one of the above-mentioned genes. In particular, this method will be used in connection with very young animals, and will comprise oral administration of the microorganism to the young animal.

Yet a further aspect of the invention provides a method of generating the live vaccine microorganisms of the first aspect comprising: (a) selecting a gut-colonising microorganism, (b) treating said microorganism so as to produce mutants in which one

or more genes associated with colonisation of the alimentary tract have been inactivated, (c) selecting and culturing a mutant with the desired properties.

Preferably the microorganisms and genes are those described  
5 above.

An animal protected as above forms a further aspect of the invention. Preferably the protected animal is a foodstuff animal, and a foodstuff substantially free of contaminating organisms prepared from a slaughtered animal as above forms a yet further  
10 aspect of the invention.

The methods and materials of the present invention will now be described, by way of illustration only, with reference to the following non-limiting examples. Other embodiments falling within the scope of the invention will occur to those skilled in the art  
15 in the light of these.

#### EXAMPLES

##### EXAMPLE 1: IDENTIFICATION OF A VARIETY OF COLONISATION GENES IN S. TYPHIMURIUM

##### MATERIALS AND METHODS

20 Chickens: all chickens were from a specified pathogen free flock. Their methods of rearing and diet have been described previously (Smith and Tucker (1975) J Hygiene, Cambridge 75: 275-292. All feed was unmedicated.

Bacterial cultures: the bacterial culture used was *S. typhimurium*  
25 F98 which was cultured as described in "Chromosomal transposon mutations affecting intestinal colonisation of chickens by *S. typhimurium*" by Barrow and Lovell in CNEVA/INRA Reports and Communications: Salmonella and Salmonellosis (September 15-17, 1992; Ploufragan/Saint-Brieuc-France), the entire technical  
30 content of which is incorporated herein by reference.

Production of transposon mutants: the method employed was that used in Barrow and Lovell (1992) supra. Briefly, mutagenesis was

carried out using Tn5 in which the kanamycin resistance gene had been replaced by that for tetracycline resistance. The transposon Tn5-Tc<sub>1</sub> was present on pCHR71 which is a thermosensitive replication mutant of the broad host range transmissible plasmid R388 encoding trimethoprim resistance. The whole plasmid, designated pCHR82, present in *E. coli* K12 strain MC1061, was used as a suicide vector for delivering the modified Tn5. The plasmid pCHR82 was transferred to *S. typhimurium* using standard broth conjugation incubating statically overnight at 30°C. After isolating the transcient on L-agar containing tetracycline and sodium nalidixate, many separate cultures of this were set up in nutrient broth containing tetracycline in the wells of a sterile haemagglutination tray incubated overnight at 43°C. Cultures from each well were tested for a loss of trimethoprim and for the maintenance of tetracycline resistance. Such cultures were considered to be transposon mutants and were stored at -70°C.

Assessment of intestinal colonisation: this was assessed as in Barrow and Lovell (1992) supra. Essentially the method used entailed the oral inoculation of individual three-week-old chickens with 0.5ml of a broth culture of the strain to be tested. Chickens were housed together. Two days later the cloaca of the chickens were swabbed and this eluted in 1 ml phosphate buffered saline. This was then plated in a standard manner (Smith and Tucker (1975) supra) on to plates of brilliant green agar (Oxoid) containing nalidixic acid (20pg per ml). Mutants of interest were retested in increasing numbers of chickens.

Assessment of virulence: newly hatched (less than 1 day old) chicks were inoculated orally with 10<sup>8</sup> c.f.u. of the strain to be tested in 0.1 ml carrier. Mortality was measured over a three week period.

Production of transductant mutant: positive results with transposon mutants were confirmed by transducing the mutated gene into a fresh parental background. The method employed was that using phage P22, as described in Barrow et al (1990) Epidemiol.

Infect. 104: 413-426, the entire technical content of which is incorporated herein by reference.

Production of defined mutants:

Results with transposon mutants and transductants were further confirmed in some cases with defined mutants, which were prepared as follows:

METHOD USED FOR *hupA*, *sipC*, *clpB* AND *rfbK*

Fragments of the genes concerned were amplified by PCR using oligonucleotide primers 1 and 2 for each gene. These were  
10 digested with *Xba*I (*clpB* primer 1, *rfbK* primer 1, and *sipC* primer 1), *Eco*RI (*clpB* primer 2, *rfbK* primer 2, *hupA* primer 1, *sipC* primer 2) or *Sal*I (*hupA* primer 2) and ligated into the suicide vector pGP704 digested with *Xba*I and *Eco*RI (for *clpB*, *rfbK*, *sipC*) or *Eco*RI and *Sal*I (for *hupA*).

15 A kanamycin resistance GenBlock (Pharmacia Biotech) was amplified by PCR using oligonucleotide primers which have *Kpn*I sites included in their 5'ends. This was ligated into the *Kpn*I sites in the cloned *rfbK*, *hupA* and *sipC* fragments of the recombinant plasmids. The kanamycin resistance GenBlock was digested from a  
20 pBluescript (Stratagene Ltd) derivative and ligated into the *Eco*RV site in the *clpB* gene fragment. *E. coli* SY327  $\lambda$  *pir* was used in the different stages of the construction, bacterial cells being made competent by treatment with 50 mM calcium chloride solution.

25 One recombinant plasmid of the correct construction was chosen for each gene concerned and was transformed into *E. coli* SM10  $\lambda$  *pir* (Simon et al (1983) Bio/Technology 1: 784-789) as described above. Defined mutations were then transferred to the wild-type *S. typhimurium* strain by conjugation; defined *S. typhimurium*  
30 mutants were selected with kanamycin, and those which showed loss of the pGP704 vector (ampicillin sensitivity) were chosen. The construction of the mutants was confirmed by PCR using primers 1

and 2 together, and in combination with the kanamycin resistance GenBlock primers.

Oligonucleotide primers for hupA (Higgins & Hillyard (1988) J. Bacteriol. 170: 5751-5758, fig 2.)

- 5 Primer 1: nucleotide positions -175 to - 154 has a natural *EcoRI* site at the 5' end

Primer 2: nucleotide positions 291 to 314 has a natural *Sall* site at the 5' end

The *KpnI* site is at nucleotide positions 142-147 in the gene

- 10 Oligonucleotide primers for sipC (DNA database entry code newembl:st25631, last updated 6<sup>th</sup> September 1995, Version 1)

Primer 1: nucleotide positions 2627-2646 *XbaI* added to 5' end

Primer 2: nucleotide positions 3600-3619 *EcoRI* added to 5' end

The *KpnI* site is at nucleotide positions 3136-3141.

- 15 Oligonucleotide primers for clpB (from *E. coli* sequence; DNA database entry code em\_ba:ecclpB last updated 23<sup>rd</sup> November 1993, Version 6)

Primer 1: nucleotide positions 1385-1405 *XbaI* site added to 5' end

- 20 Primer 2: nucleotide positions 2625-2645 *EcoRI* site added to 5' end

The *EcoRV* site is at nucleotide positions 1909-1914

Oligonucleotide primers for rfbK (DNA database entry code em\_ba:serfbb last updated 23<sup>rd</sup> November 1993, Version 1)

- 25 Primer 1: nucleotide positions 18,678-18,698 *XbaI* site added to 5' end

Primer 2: nucleotide positions 19,722-19,741 *EcoRI* site added to 5' end

The *KpnI* site is at nucleotide positions 19,075-19,080

Kanamycin cassette oligonucleotides

5 Primer 1 GAATTCGGTACCCGCTGAGGTCTGCCTCGTGAAGG

Primer 2 GAATTCGGTACCAAAGCCACGTTGTGTCTAAAATC

RESULTS

The results are shown in Table 1 below.

The mutated genes are as follows:

- 10 *hupA*: this gene encodes a polypeptide of the nucleoprotein HU which is involved in maintaining the structure of the nucleoid. It is possible that HU, as with other nucleoproteins, effects changes in gene expression through changes in DNA supercoiling (see Higgins & Hillyard (1988) J. Bacteriol 170: 5751-5758).
- 15 *dkSA*: in *E. coli* it was found that, at high copy number, the *dkSA* gene suppressed some of the mutant phenotypes associated with the *dnaK*, *dnaJ* and *grpE* genes which are involved in control of the heat shock response (see Kanji & Craig (1990) J. Bacteriol 172: 2055-2064). The *S. typhimurium dkSA* mutant does not grow on
- 20 minimal medium but requires the addition of amino acids.



Table 1

Mutation		Mortality measured by virulence test	Colonisation phenotype of mutant	Colonisation phenotype of transducant	Colonisation phenotype of mutant
1	Wild type	20/22 (10/10)	-	-	-
2	<i>hupA</i>	3/21 (5/31)	poor	poor	poor
3	<i>dkaA</i>	2/21 (2/30)	poor	poor	n/d
4	<i>sipC</i>	2/23 (2/33)	poor	n/d	n/d
5	<i>clpB</i>	2/22	poor	as wild type	n/d
6	<i>rfaY</i>	19/22	poor	poor	n/d
7	<i>rfaK</i>	0/23	poor	n/d	n/d
8	<i>rfaB</i>	3/23	poor	n/d	n/d
9	<i>rfaK</i>	5/21 (1/33)	poor	n/d	n/d

The figures in brackets show the results in a subsequent mortality trial using oral administration of a larger batch of newly hatched chicks.

*sipC*: the gene product is required for Salmonella invasion of host cells (see Kaniga et al (1985) J. Bacteriol. 177: 3965-3971).

*clpB*: the *E. coli* gene product alters the specificity of the ClpP protease. In addition, ClpB protein possesses chaperone activity,

and is probably expressed during the heat shock response (see Kitagawa et al (1991) J. Bacteriol. 173:4247-4253).

*rfa* and *rfb* genes: these genes are required for the synthesis of lipopolysaccharide (LPS). The role of the *rfaY* gene product is not known, but it is thought that it may regulate activity of RfaJ. The *rfaY* mutant shows normal LPS with SDS-PAGE, while the *rfaK*, *rfbB* and *rfbK* mutants show the rough phenotype (see MacLachlan et al (1991) J. Bacteriol. 173:7151-7163 for *rfa* genes; Jiang et al (1991) Mol. Microbiol. 5:695-713 for *rfb* genes).

#### EXAMPLE 2: PREPARATION OF LIVE VACCINE ORGANISM

- 10 A strain is selected which has a high invasiveness and the ability to demonstrate a specific competitive exclusion effect. A specific attenuating, colonisation-impairing mutation (e.g. in *clp B*) is introduced. Alternatively a non-colonising mutation, not associated with attenuation, is introduced into the strain
- 15 which has been mutated in some other way. A negative serological marker (e.g. non-flagellation or roughness) is also introduced.

#### EXAMPLE 3: PREPARATION OF VACCINE

- The vaccine strain is suspended in a storage medium such as are well known to those skilled in the art, or lyophilised for
- 20 reconstitution prior to use.

#### EXAMPLE 4: INOCULATION PROTOCOL

- For day-old chicks the vaccine is used as a spray such that each bird receives at least  $10^6$  c.f.u. If breeding or layer birds are being vaccinated a second parenteral vaccination is given at the
- 25 age of 12-14 weeks consisting of  $10^7$  c.f.u. in 0.1 ml given intra-muscularly or subcutaneously.

CLAIMS

1. A live microorganism for use as a vaccine, said microorganism comprising a gut-colonising microorganism which is capable of evoking a protective immune response in an animal to which it is administered, characterised in that the microorganism is a mutant organism whose ability to colonise the alimentary tract of said animal is inhibited as a result of a mutation.
2. A microorganism as claimed in claim 1 wherein mutation is such that, as a result of its presence, the time scale over which the microorganism is excreted from the alimentary tract of the animal following inoculation is reduced by at least 50%.
3. A microorganism as claimed in claim 1 or claim 2 wherein the mutation is such that, as a result of its presence, the quantity of microorganism is reduced to less than 20% of that found with a similar organism without said mutation, as measured 3 weeks after inoculation.
4. A microorganism as claimed in any one of the preceding claims wherein the microorganism is an Enterobacteriaceae.
5. A microorganism according to claim 4 selected from the group: *Salmonella*, *E. coli*, *Yersinia*, *Campylobacter*, *Listeria*, *Bacillus cereus* or *Shigella*.
6. A microorganism according to claim 5 which is a *Salmonella*.
7. A microorganism as claimed in any one of the preceding claims wherein the mutation results in the down-regulation or inactivation of one or more genes associated with colonisation of the alimentary tract of the animal.
8. A microorganism as claimed in claim 7 wherein said gene associated with colonisation of the alimentary tract is *hupA*, *dkxA*, *rfaY*, *sipC* or *clpB*.
9. A microorganism as claimed in any one of the preceding claims wherein said microorganism has at least one further mutation which attenuates said organism.

10. A microorganism as claimed in claim 7 wherein the said further mutation comprises one or more mutations in the *aro A*, *gal E* or *pur A* or in an electron transport gene.

11. A microorganism as claimed in any one of the preceding claims wherein the microorganism expresses heterologous antigens.

12. A microorganism as claimed in any one of the preceding claims wherein the microorganism further comprises a negative serological marker.

13. A microorganism as claimed in claim 12 wherein the negative serological marker is selected from the group: roughness, non-flagellate, non-fimbriate, mutated SEFA.

14. A mutant gut-colonising microorganism wherein at least one of the genes selected from *hupA*, *dkxA*, *rfaY*, *sipC* or *clpB* has been down-regulated or inactivated so as to inhibit the ability of the microorganism to colonise the alimentary tract of an animal possessing a normal adult gut flora.

15. A mutant microorganism according to claim 14 which comprises a mutant *Enterobacteriaceae*.

16. A mutant microorganism according to claim 14 which is selected from *Salmonella*, *Yersinia*, *E. coli*, *Campylobacter*, *listeria*, *Bacillus cereus* or *Shigella*.

17. A mutant microorganism according to any one of claims 14 or claim 16 which comprises further mutations which attenuate the microorganism and/or provide a negative serological marker.

18. A mutant microorganism according to claim 17 wherein the said further mutations one or more mutations in the *aro A*, *gal E*, *pur A* or an electron transport gene.

19. A vaccine comprising a microorganism according to any one of the preceding claims in combination with a pharmaceutically acceptable carrier or diluent.

20. A vaccine according to claim 19 dosage unit form.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01932

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N1/20 A61K39/02 A61K39/112 A61K39/108 A61K39/106  
A61K39/07 //(C12N1/20,C12R1:01),(C12N1/20,C12R1:19),(C12N1/20,  
C12R1:42),(C12N1/20,C12R1:085)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US IBA A M ET AL: "Interference between Salmonella serotypes in intestinal colonisation of chickens: Correlation with in vitro behaviour." XP002044731 see abstract &amp; FEMS MICROBIOLOGY LETTERS 131 (2). 1995. 153-159,</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1,19,20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

28 October 1997

Date of mailing of the international search report

11. 11. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

# INTERNATIONAL SEARCH REPORT

Inte. onal Application No

PCT/GB 97/01932

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US  BERCHIERI A JR ET AL: "FURTHER STUDIES ON  THE INHIBITION OF COLONIZATION OF THE  CHICKEN ALIMENTARY TRACT WITH  SALMONELLA-TYPHIMURIUM BY PRE-COLONIZATION  WITH AN AVIRULENT MUTANT."  XP002044732  see abstract  &amp;  EPIDEMIOL INFECT 104 (3). 1990. 427-442,  ---</p>	1,19,20
A	<p>US 5 389 368 A (GURTISS R.) 14 February  1995  see the whole document  ---</p>	1-20
A	<p>WO 95 24475 A (SECR DEFENCE BRIT ;TITBALL  RICHARD WILLIAM (GB); WILLIAMSON ETHEL) 14  September 1995  see the whole document  ---</p>	1-20
P,X	<p>METHNER U. ET AL.: "Comparative study of  the protective effect against Salmonella  colonisation in newly hatched SPF chickens  using live, attenuated Salmonella vaccine  strains ..."  INTERNATIONAL JOURNAL OF FOOD  MICROBIOLOGY,  vol. 35, no. 3, 15 April 1997,  pages 223-230, XP002044730  see the whole document  -----</p>	1-20

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 97/01932

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5389368 A	14-02-95	US 5468485 A	21-11-95
		US 5387744 A	07-02-95
		US 5294441 A	15-03-94
		AU 1955088 A	04-01-89
		CA 1338957 A	04-03-97
		CN 1030018 A	04-01-89
		DE 3886506 D	03-02-94
		DE 3886506 T	19-05-94
		DK 52789 A	03-02-89
		EP 0315682 A	17-05-89
		IE 63905 B	14-06-95
		JP 1503442 T	22-11-89
		WO 8809669 A	15-12-88
-----			
WO 9524475 A	14-09-95	AU 1853995 A	25-09-95
		CA 2184902 A	14-09-95
		EP 0753061 A	15-01-97
-----			